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Identification of a His54Gln Substitution in von Willebrand Factor From a Patient With Defective Binding of Factor VIII

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A patient with type 2N ("Normandy" variant) von Willebrand's disease is described. Her von Willebrand factor level was borderline low, while her factor VIII was markedly decreased to 7%. Her plasma von Willebrand factor demonstrated a decreased ability to complex with factor VIII in vitro, binding less than 10% when compared to normal plasma von Willebrand factor. The factor VIII released into the circulation after the patient received DDAVP had a shortened survival in vivo. Nucleotide sequence analysis revealed a T-to-A transition at nucleotide 2451 on both alleles. This transition results in a substitution of Gln for His at amino acid 54 in the mature subunit of von Willebrand factor. © 1996 Wiley-Liss, Inc.*

Key words: von Willebrand factor, Normandy variant, genetic defect, factor VIII binding defect

INTRODUCTION

von Willebrand factor (vWf) is an important hemostatic protein that functions as a carrier for factor VIII in plasma [1,2] and that also performs essential functions for normal platelet adhesion and aggregation [3]. As a part of its carrier function, vWf forms a noncovalent complex with factor VIII [4,5] that increases the half-life of factor VIII in the circulation and protects it from proteolysis by activated protein C and factor X_a [6–9].

The binding region for factor VIII in the vWf molecule resides within the N-terminal 272 residues [10,11], and an epitope important in this binding has been localized to a region that includes Thr78 and Thr96 [12]. Individuals with a binding defect for factor VIII have been reported, and their mutations have been localized to several different sites within the coding region for the first 91 amino acids of vWf [13]. Patients generally present with a hemophilia-like clinical and laboratory picture manifested by a decreased factor VIII level; their vWf level may be normal, or may be decreased if the patient has coinherited a defect for type 1 von Willebrand's disease (vWd) [14,15]. These patients are often referred to as a "Normandy" variant of von Willebrand's disease and are now classified as type 2N vWd [16]. Two heterozygous patients with a substitution of His54Gln have been reported [17,18]; one also has a second substitution, Arg19Trp, on the same allele [17]. We now report a patient with a homozygous His54Gln substitution who has no other mutations within the coding region for the first 100 amino acids.

MATERIALS AND METHODS

Case Study

The patient is a 41-year-old African American woman with lifelong symptoms of easy bruising, mild epistaxis, and menorrhagia. She had one pregnancy which was delivered by cesarean section for obstetrical reasons and was not reported to be complicated by bleeding. The menorrhagia lead to a hysterectomy at age 32, and the surgery was complicated by postoperative bleeding, reexploration of the wound site, and transfusion of 13 units of blood products. The family history is positive for bleeding in one of the patient's sisters and questionable in the mother; it is negative in the father, the remaining four siblings, and the patient's one son (age 14 years). The patient's laboratory studies are noted in Table I; results are also shown following a preoperative trial of DDAVP (0.3 μg/kg).

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TABLE I. Laboratory Evaluation of the Patient at Baseline and After DDAVP (0.3 μg/kg)

		After DDAVP			
	Baseline	30 min	2 hr	4 hr	Normal
Factor VIII	7%	38%	19%	14%	50–150%
Factor VIII Ag	6%	55%	21%	20%	44–145%
vWf Activity	41%	91%	86%	81%	44–148%
vWf Ag	45%	93%	87%	94%	50–150%
Multimers	WNL	↑	↑	↑	
Platelet vWf activity	32%				42–144%
Platelet vWf Ag	10%				9–48%
Bleeding time	4.5 min				2.5–9.0 min

WNL, within normal limits; ↑, increase in the density of multimers and the presence of high-molecular-weight multimers.

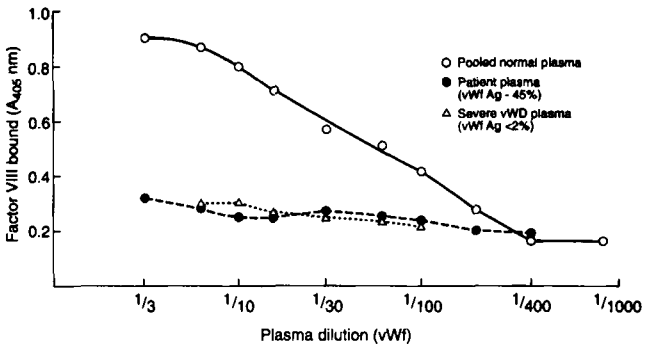

Fig. 1. Dilutions of plasma from patient, normal, and severe vWd patient (no measureable vWf) were bound to microtiter plates; after separation of endogenous plasma factor VIII, purified factor VIII was added, and its binding was assessed by chromogenic assay for factor VIII.

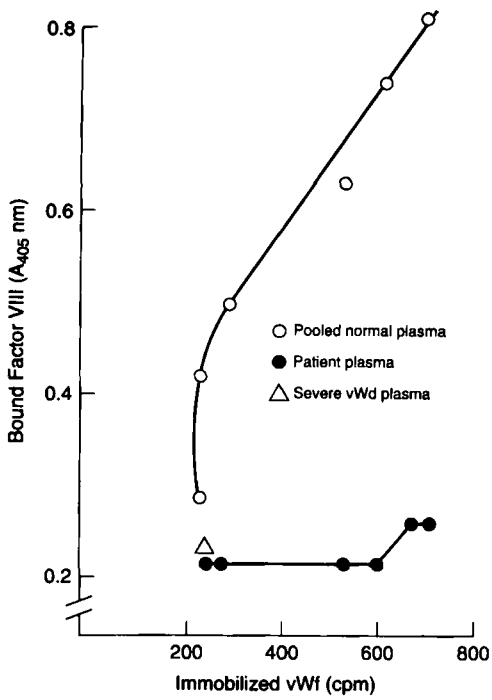

Fig. 2. See legend to Figure 1. After assessing factor VIII activity, the initially bound vWf was quantified by measuring binding of a second antibody to vWf.

Materials and Methods

Factor VIII and vWf assays. Factor VIII function was measured in a one-stage clotting assay using human factor VIII-deficient substrate plasma [19]. Factor VIII antigen was measured in a radioimmunoassay using a monospecific antibody from a patient with a factor VIII inhibitor [20]. Ristocetin cofactor activity was assessed in a formalin-fixed platelet assay that uses a visual endpoint [21], and vWf antigen was measured in a radioimmunoassay using a polyclonal rabbit antibody [22]. vWf multimers were analyzed by standard methods [23,24].

Binding assay of purified factor VIII to vWf. The ability of patient or normal plasma vWf to bind factor VIII was measured by a modification of the method of Nishino et al. [25]. Microtiter plates were coated for 2 hr at room temperature and then overnight at 4°C with 100 μl of a rabbit polyclonal antibody to vWf (10 μg/ml) (Dako Corp., Carpinteria, CA). Except where noted, further incubations were performed at 37°C. After washing with veronal-buffered saline (VBS, barbital 0.015 M, Na barbital 0.01 M, NaCl 0.125 M, $CaCl_2$ 2 mM), the wells were blocked with 2% gelatin for 60 min. After washing with VBS, dilutions (100 μl) of normal or patient plasma in VBS were added to the wells and incubated for 60 min. Plasma factor VIII bound with the vWf was removed by the addition of 400 mM Ca^{2+} in 0.05 M imidazole buffer, pH 6.8 for 20 min. After washing with VBS, goat antirabbit IgG was added at a concentration of 0.5 mg/ml for 30 min. The wells were washed with VBS, and purified factor VIII (Recombinate, Hyland Div, Baxter, Glendale, CA) was added to the wells at a concentration of 1.0 U/ml and incubated for 20 min. After the wells were washed with VBS, the bound factor VIII was measured with a chromogenic assay (Coatest Factor VIII, Kabi, Helena Lab, Beaumont, TX), modified from the directions provided with the kit so as to use only 1/4 vol of the reagents provided. The activity was measured by absorbance at 410 nm. The original quantity of plasma vWf bound in the wells was assessed by adding a second polyclonal antibody to vWf, which was labeled with ^{125}I.

DNA isolation and PCR amplification. DNA was isolated from peripheral blood leukocytes by standard methods after lysis by Triton X-100 [26]. PCR amplification was carried out on genomic DNA using Taq DNA polymerase provided with the GeneAmp PCR Reagent kit (Perkin Elmer Cetus, Norwalk, CT); reactions were carried out according to the kit instructions in an automated thermocycler. Thirty cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C were completed. Primers

included vWf sense primer 1, intron 17, 15/In22-In43 (5'-ACAGACTCTAGGGGACCAAAGG-3') and antisense primer 1, intron 18,15/In413-In392 (5'-AGAAAACT-GAAGGGCAGGCACC-3'); vWf sense primer 2, intron 18, 16/In24-In43 (5'-GTGGCCCTGAGGACTTTTGG-3') and antisense primer 2, intron 19:exon 19, 16/In6-In1:a2551-2537 (5'-ACTGAGCCTCACCAAGTGTTG-C-3'); sense primer 3, intron 19, 17/In92-In113 (5'-CTAT-GAAGTCCACACTCCACGC-3') and antisense primer 3, intron 20, 17/In490-In464 (5'-TTCTGCAGACAGAT-CCACAGAACCCAA-3') [27]. Amplified fragments were visualized by staining with ethidium bromide after electrophoresis in 2% agarose gels. The fragments were isolated by preparative electrophoresis and recovered using Geneclean (Bio 101, La Jolla, CA).

DNA sequencing. Sequencing of the amplified fragments was performed by the dideoxy method of Sanger using T7 DNA polymerase and the protocol and other reagents provided with the Sequenase kit, version 2 (United States Biochemical, Cleveland, OH). ^{35}S-deoxyadenosine 5'-triphosphate was used for labeling. Single-stranded DNA was prepared by asymmetric PCR [28].

Restriction enzyme analysis. Digestion of the PCR fragment 2 containing the transition was carried out at 37°C for 1 hr, using *Nla*III (New England Biolabs, Beverley, MA) at a concentration of 1 U/10 μl PCR product, and fragments were separated by electrophoresis in agarose gels. Amplified DNA from 30 normal subjects was analyzed in the same way.

RESULTS

Clinical Studies

Table I shows borderline low values for the patient's vWf activity and antigen (mean of three measurements); the distribution of vWf multimers, however, was normal (data not shown). The level of factor VIII (mean of three measurements) is decreased out of proportion to the vWf levels, resulting in a factor VIII/vWf ratio of 0.16. Following the infusion of DDAVP (Table I), there is an initial appropriate rise in factor VIII, but the half-life of factor VIII is decreased to less than 50% when compared with the half-life of vWf (at 2 hr after DDAVP, the factor VIII activity is 19%, while the vWf activity is 86%). Figure 1 compares the binding of purified normal factor VIII to patient and normal vWf and indicates a binding of less than 10% to the patient's vWf as compared to normal. Further studies using a radiolabeled antibody to vWf verified that the patient's (or control) vWf had bound to the plate in the initial step of the assay (Fig. 2).

DNA Sequence

Figure 3 shows a T-to-A transition at nucleotide 2451 in codon 817 of the mature vWf subunit. No other abnormalities were found in the region coding for the first 100 amino acids of the mature subunit, including amino acid 19, and no evidence for heterozygosity was found.

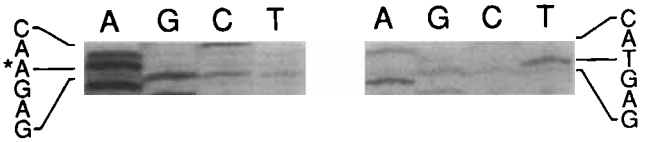

Fig. 3. DNA sequence analysis of patient (left) and normal control (right) DNA indicating T-to-A transition.

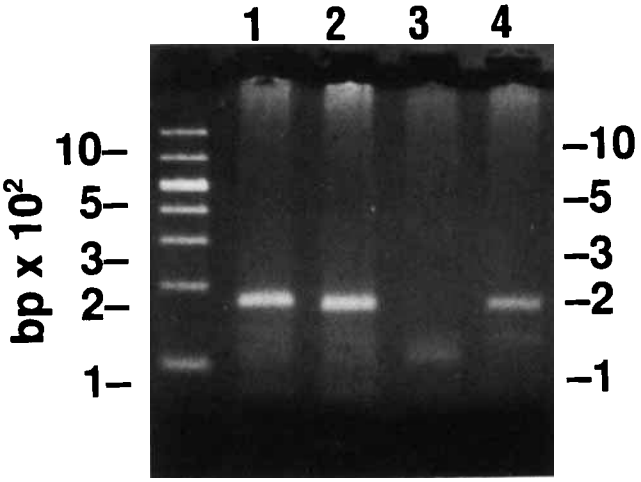

Fig. 4. Analysis of patient and normal PCR fragment 2 after incubation with *Nla*III. Lanes 1, 2, contain patient DNA with and without *Nla*III, respectively; lanes 3, 4, contain normal DNA incubated with and without *Nla*III, respectively. The band at 130 bp represents a cleavage product of *Nla*III; the slightly larger band faintly visible in all lanes is not identified.

NlaIII Restriction Analysis

Since the T-to-A transition causes the loss of a cleavage site for *Nla*III, the patient's fragment 2 and that of 30 normal subjects were analyzed by agarose gel electrophoresis after incubation with *Nla*III; all the normal subjects showed the expected restriction fragment pattern, whereas the patient's DNA was not cleaved. A representative gel is shown in Figure 4.

DISCUSSION

The patient has a lifelong bleeding disorder of moderate severity with slightly low vWf levels and very low factor VIII levels. The laboratory studies indicate a binding defect of the patient's vWf for normal factor VIII; this was also evidenced in vivo by the rapid clearance of factor VIII after infusion of DDAVP. Although there is no evidence for a co-inherited type 1 defect that could account for the borderline level of vWf in our patient, we cannot rule out this possibility on the basis of our

studies. It is also possible that the mutation at codon 817 affects synthesis or secretion of the patient's vWf. The inheritance pattern is uncertain because relatives have been unavailable for study, but phenotypically it appears that two of the six siblings are affected. The patient's mother died during childbirth, and no details are known about the cause of her death.

The response to DDAVP shows a five-fold rise in the patient's factor VIII, but a shorter than normal half-life in the circulation. This result is anticipated because of the increased clearance of factor VIII which is not bound to vWf [2], and this pattern has also been documented in several families with type 2N vWd [15].

The DNA sequencing studies indicate a T-to-A transition at nucleotide 2451, which codes for the nonconservative substitution of glutamine for histidine at amino acid 54 in the mature subunit of vWf. The patient appears to be homozygous for this defect and represents the first example of this mutation in the homozygous state associated with defective binding of factor VIII. Two other patients have been reported who are heterozygous for this mutation and have defective binding of factor VIII; each has a second defect in the vWf gene: Kroner et al. [17] described a patient with a His54Gln substitution who has a second defect, Arg19Trp, on the same allele. In addition, this patient has also co-inherited a defect on his other allele that leads to a translational stop codon. A second patient with a heterozygous His54Gln substitution also has an additional defect leading to a different type of variant vWD, a type 2B defect. This patient was reported to show normal binding of his plasma vWf for factor VIII [18]. It is possible that the normal level of binding is due to the second allele, which displays normal sequence in the amino terminus.

Four other point mutations have been described in the other reported patients with binding defects for factor VIII: Arg19Trp, Thr28Met, Arg53Trp, and Arg91Gln [17, 29–35]. All the patients have either been homozygous for a given defect or have coinherited a second defect for type 1 vWD on their other allele, which causes markedly decreased expression.

Impaired binding of factor VIII by recombinant proteins containing mutations at Thr28, Arg53, and Arg91 has been documented [31,36,37] and the recombinant vWf, which contains both the Arg19Trp and the His54Gln mutations shows decreased binding of factor VIII [17]. Site-directed mutagenesis of vWf, which directs the nonconservative change of each charged amino acid in the region Arg5 to Asp133 to an alanine, revealed that substitution of Glu24, Glu55, and Arg57 by Ala impairs the binding of factor VIII [38]. Studies in which Asn94, an N-glycosylation site, was mutated to Gln revealed a decrease in the binding of factor VIII to the altered vWf of approximately 50% [39]. Interestingly, a patient who has a substitution of Ser for Asn94 (in the complex setting of a second defect causing a type 2 vWd) has normal binding of his plasma vWf for factor VIII [18]. These reports, together with the data provided in this study, clearly indicate that several amino acids in the N-terminal region of vWf are important for the binding of factor VIII to vWf. In addition, such binding is dependent on the presence of charged amino acids as well as oligosaccharide side chains.

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